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Molecular Dynamics of Detoxification and Toxin-Tolerance Genes in Brown Planthopper (*Nilaparvata lugens* Stål., Homoptera: Delphacidae) Feeding on Resistant Rice Plants

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To investigate the molecular response of brown planthopper, *Nilaparvata lugens* (BPH) to BPH-resistant rice plants, we isolated cDNA fragments of the genes encoding for carboxylesterase (CAR), trypsin (TRY), cytochrome P450 monooxygenase (P450), NADH-quinone oxidoreductase (NQO), acetylcholinesterase (ACE), and Glutathione S-transferase (GST). Expression profiles of the genes were monitored on fourth instar nymphs feeding on rice varieties with different resistance levels. Northern blot hybridization showed that, compared with BPH reared on susceptible rice TN1, expression of the genes for P450 and CAR was apparently up-regulated and TRY mRNA decreased in BPH feeding on a highly resistant rice line B5 and a moderately resistant rice variety MH63, respectively. Two transcripts of GST increased in BPH feeding on B5; but in BPH feeding on MH63, this gene was inducible and its expression reached a maximum level at 24 h, and then decreased slightly. The expression of NQO gene was enhanced in BPH on B5 plants but showed a constant expression in BPH on MH63 plants. No difference in ACE gene expression among BPH on different rice plants was detected by the RT-PCR method. The results suggest these genes may play important roles in the defense response of BPH to resistant rice. Arch. Insect Biochem. Physiol. 59:59–66, 2005.

KEYWORDS: detoxification and toxin-tolerance gene; brown planthopper; rice; RT-PCR; northern hybridization

INTRODUCTION

The brown planthopper (*Nilaparvata lugens* Stål., Homoptera:Delphacidae, BPH) is one of the most destructive pests of rice (*Oryza sativa* L.) throughout Southeastern and Eastern Asia. It sucks sap from rice phloem, which impacts plant growth and development, even results in hopperburn (Rubia-Sanchez et al., 1999). At present, spraying chemical insecticides is a common practice to control BPH, which provides short-term control but causes the insect resurgence in most cases of improper use. The susceptibility of currently planted rice varieties has been considered as the cause of the widespread infestations of this insect. Growing resistant rice varieties has proven to be an effective measure in integrated pest management (IPM) systems (Cuong et al., 1997; Sogawa et al., 2003).

Efforts have been made over the years to study the feeding behavior and physiology response of BPH on rice varieties with different resistance levels and the effect of host resistance on the growth, development, and reproduction of the insect. Studies revealed that, when reared on susceptible rice

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Abbreviations used: ACE = acetylcholinesterase; BPH = brown planthopper; CAR = carboxylesterase, GST = Glutathione S-transferase; NQO = NADH-quinone oxidoreductase; P450 = cytochrome P450 monooxygenase; RT-PCR = reverse transcription-polymerase chain reaction; TRY = trypsin.

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varieties, BPH insects increase egg laying, and eggs and nymphs have a high survival ratio; but on resistant rice varieties, the survival ratio of eggs and nymphs is significantly lower, nymph development generally retarded, and population growth effectively suppressed (Khan and Saxena, 1988; Denno and Roderick, 1990). It is clear that the behavior, growth, development, and reproduction of BPH are profoundly influenced when feeding on the resistant plants. However, the molecular response of BPH to ingestion of different rice varieties has not been elucidated yet.

Numerous studies revealed that plants constitutively or inductively produce the defense proteins and toxic phytochemicals that deter, poison, or starve herbivores that feed on them. Herbivorous insects can responsively counteract defensive proteins and detoxify toxic allelochemicals in plant diet, by regulating expression of genes coding for cytochrome P450 monooxygenases (P450s), carboxylesterases (CARs), Glutathione S-transferases (GSTs), acetylcholinesterase (ACE), NADH-quinone oxidoreductase (NQO), and trypsin (TRY) (Glendinning and Slansky, 1995; Schuler et al., 1996; Gatehouse et al., 1997; Mazumdar-Leighton et al., 2001). The present study focuses on determining whether the expressions of the six genes are altered after BPH insects ingest resistant rice plants. The results revealed that five of the genes were regulated by one or both of the resistant rice varieties.

MATERIALS AND METHODS

Insects and Rice Varieties

The brown planthopper insects (biotype II) were reared on plants of susceptible rice TN1 at 25 ± 2°C, 80% relative humidity, under long photoperiod conditions (16-h light/8-h dark cycles). For feeding experiments, the seeds of TN1 and two BPH-resistant rice varieties B5 and MH63 were planted in plastic pots (Shi et al., 2003; Ren et al., 2004). At the 3-leaf stage of the seedlings, the forth instar nymphs of BPH were collected and transferred onto the seedlings and maintained for 6, 12, 24, 48, and 72 h. The insects were collected at fixed intervals and immediately frozen in liquid nitrogen for RNA extraction. The fourth instar nymphs at the start of the feeding experiments (0 h) were used as control (C).

Amplification and Cloning of cDNA Fragments

Total RNA was isolated from BPH nymphs feeding on TN1 plants by using TRIzol reagent (Invitrogen, La Jolla, CA). RT-PCR reactions were carried out by using the Access RT-PCR System Kit (Promega, Madison, WI), with conditions and degenerate primers for CAR, TRY, P450, NQO, ACE, and GST genes according to previous reports (Yano et al., 1997; Huang et al., 1998; Jamroz et al., 2000; Kasai et al., 2000; Mazumdar-Leighton et al., 2001; Gao et al., 2002). The sequences of forward primers and reverse primers are listed in Table 1. The amplification products were fractionated in a 0.7% agarose gel. The desired DNA bands were recovered and cloned into pGEM-T vector (Promega), and the ligated products were transformed into DH5α *Escherichia coli* competent cells.

Sequencing the cDNA Fragments

The inserted cDNA fragments were sequenced at both ends by using universal T7 and M13 primers and BigDye™ terminator cycle sequencing version 2.0 ready kit (PE Applied Biosystems, Foster City, CA), on an ABI 377 automatic sequencer (PE Applied Biosystems). The sequences were compared against all databases in GenBank by using the BLAST server command "blastx."

Northern Blot Hybridization and RT-PCR Analysis

For Northern blot hybridization, 20 μg of total RNAs from each stored BPH sample were electrophoresed on formaldehyde denatured agarose gels (1.5%). RNAs were blotted onto a Hybond-N^+ (Amersham, Arlington Heights, IL) nylon membrane and hybridized with the cDNA fragments labeled with [α-^{32}P]-dCTP (Perkin Elmer Life Sciences). The membranes were hybridized overnight at 65°C, washed in 1 × SSC, 0.2% (W/V) SDS at 65°C for

TABLE 1. Primers Used for PT-PCR Reactions*

Primer	Sequence (5'-3')
P450-F	CGGAATTCGA(A/G)AC(A/G/C/T)(A/C/T)(C/T)(A/G/C/T)(A/C)G(A/G/C/T)CC(A/G/C/T)(G/T)C
P450-R	CGGAATTCGG(A/G/C/T)CC(A/G/C/T)(G/T)C(A/G/C/T)CC(A/G)AA(A/G/C/T)GG
GST-F	ATGAA(A/G)(T/C)TNTA(T/C)AA(A/G)(T/C)TNGA(T/C)ATG
GST-R	(A/G)TACTCNGGCGTCGTGTG(A/G)TC
CAR-F	TGGATTTAYGGNGGNGG
CAR-R	CCNGCRCTYTCNCCRTT
TRY-F	ATTGTGACCGCCGCTCA(C/T)TG
TRY-R	TGGGCCACCGGA(A/G)TC(A/C/G/T)CC(C/T)TG
NQO-F	ATCTG(C/T)GG(A/G/C/T)GA(A/G)GA(A/G)AC
NQO-R	TC(C/G)C(G/T)(A/G)CA(A/G/C/T)GG(C/G)GT(A/G)CA
ACE-F_0	GGAATTCGA(AG)ATGTGGAA(TC)CC(TCAG)AA(TC)
ACE-F_1	GGAATTCTGGAT(TCA)TA(TC)GGIGG(TCAG)GG
ACE-R	GGAATTCCIGCI(GC)(AT)(TC)TC(TCAG)CC(AG)AA
rACE-F	GTACGATGCTGACATGGTGG
rACE-R	AATGTTGTCCTTGAGCCAGC

*F, R, F_0, and F_1 represent forward primer, reverse primer, forward outer primer, and forward inner primer, respectively.

15 min, and in 0.5 × SSC, 0.1% (W/V) SDS at 65°C for another 15 min, then exposed to X-ray films (FUJI medical X-ray films, Japan) at –20°C with an intensifying screen.

RT-PCR reaction for verifying ACE gene expression was performed by using the Access RT-PCR System Kit (Promega) and the conditions were 48°C for 45 min, 94°C for 3 min, 28 cycles of 94°C for 30 sec, 55°C for 1 min, and 68°C for 1.5 min, then 68°C for 7 min and kept at 4°C. The primer pairs were rACE-F and rACE-R, listed in Table 1. The conditions for amplifying a 200-bp cDNA fragment of the actin gene were the same as above except that 24 cycles were used.

RESULTS

In this work, we performed RT-PCR to clone cDNA fragments of CAR, TRY, P450, NQO, ACE, and GST genes from BPH. The amplified products of expected sizes are shown in Figure 1. Products for the first five genes appeared as a single clear band, except the GST gene, which showed a smear containing 3–4 main bands; the smallest one was comparable to the expected size (Huang et al., 1998). In Figure 1, the band of ACE gene was the product of the second amplification reaction of semi-nested PCR (Gao et al., 2002). The six DNA bands (Fig. 1, arrowheads) were sliced from gel and cloned into pGEM-T vector for sequencing.

The sequencing results showed that lengths of the cDNA fragments were 396 bp (CAR), 453 bp (TRY), 237 bp (P450), 538 bp (NQO), 278 bp (ACE), and 201 bp (GST), respectively. Homology analysis revealed that the deduced amino acids of the six cDNA fragments had a high similarity (100, 100, 58, 76, 88, and 100%) to those of the CAR gene in *Nilaparvata lugens* (AC: AF302777), TRY gene in BPH (AC: AJ316142), P450 gene in *Lygus lineolaris* (AC: AY125086-1), NQO gene in *Bos Taurus* (AC: AY051642-1), ACE gene in *Nephotettix cincticeps* (AC: AF145235-1), and GST gene in *Nilaparvata lugens* (AC: AF448500), confirming the amplified products were parts of the desired genes (Table 2). The sequences of the six cDNA fragments have been submitted to the EMBL nucleotide database library and the accession numbers are listed in Table 2.

Northern hybridization analysis was carried out to study the expression profiles of the genes for CAR, TRY, P450, NQO, and GST. To exclude the possibility of developmental regulation, expression of the genes in control insects feeding on the susceptible rice TN1 at all time points has been included. The five genes varied in intensity and timing of expression (Fig. 2). Four genes except P450 showed a constant pattern in BPH feeding on TN1 during the time course and in control, while the P450 gene mRNA could not be detected by Northern blot analysis probably for its relatively low expression level in such a case. In BPH feeding on rice plants of B5 and MH63, the expression

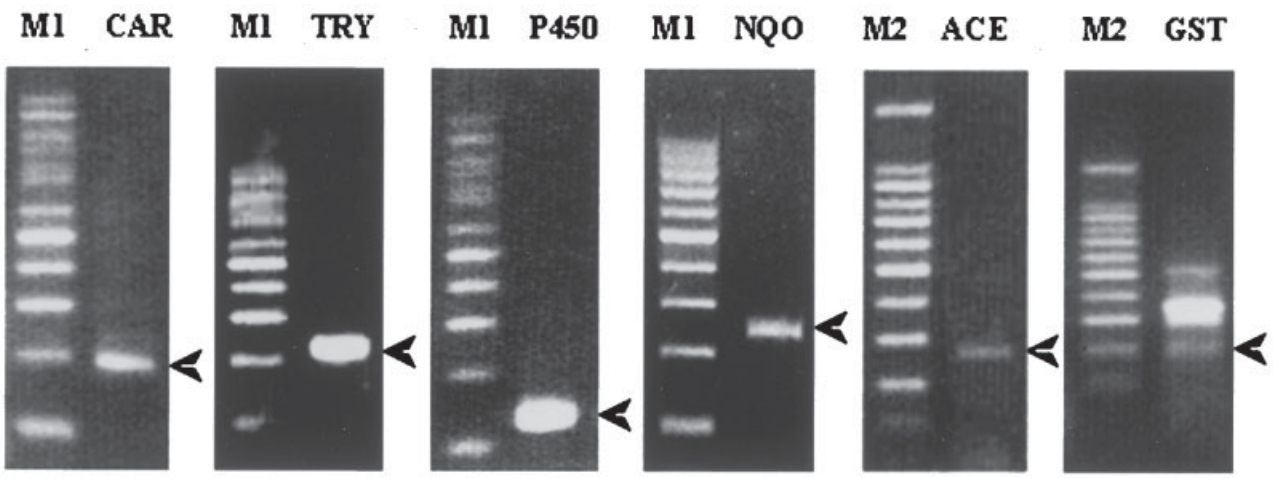

Fig. 1. Amplified products of CAR, TRY, P450, NQO, ACE, and GST genes in brown planthopper. The products were separated in a 0.7% agarose gel and stained with ethidium bromide. Arrowheads correspond to cDNA fragments of interest. M1 and M2 indicate 200-bp and 100-bp DNA ladder, respectively.

of P450 gene and CAR gene was up-regulated and the expression of TRY gene down-regulated compared with the controls. P450 gene transcripts accumulated rapidly and kept constant from 6 to 72 h, while the two transcripts of CAR gene showed slowly increased accumulation. Up-regulation of NQO gene was confirmed in BPH on B5, but this gene retained a constant expression level in BPH on MH63. GST gene also had two transcripts. Both the transcripts had an increased intensity pattern in BPH on B5. However, in BPH on MH63, the gene expression level increased slowly and reached a peak at 24 h, then decreased slightly. We failed to detect the transcripts of the ACE gene by Northern analysis. It is assumed that the expression level of this gene was lower than the expression level that could be detected by Northern hybridization technique. The RT-PCR method was employed to verify this gene expression, and the result indicated there was no alteration in ACE gene expression in BPH feeding on both resistant rice plants (Fig. 3).

DISCUSSION

In this study, the cDNA fragments of six genes have been cloned by a degenerate RT-PCR strategy (Fig. 1). The deduced amino acids of these cDNA fragments have a high homology with part of the CAR gene, TRY gene, P450 gene, NQO gene, ACE gene, and GST gene. The cDNA fragments of P450

TABLE 2. Molecular Characterization of cDNA Clones Obtained by Degenerate RT-PCR in Brown Planthopper

Clone	Accession no.	Length (bp)	Homology	Amino acid identity	E value
CAR	AJ629017	396	Carboxylesterase precursor protein (*Nilaparvata lugens*)	131/131 (100%)	0
TRY	AJ629011	453	Trypsin-like protease protein (*Nilaparvata lugens*)	151/151 (100%)	0
P450	AJ629019	237	Cytochrome P450 monooxygenase CYP6XIv3 protein (*Lygus lineolaris*)	45/78 (58%)	7e-22
NQO	AJ629454	538	NADH dehydrogenase protein (*Bos taurus*)	136/180 (76%)	2e-75
ACE	AJ629025	278	Acetylcholinesterase precursor protein (*Nephotettix cinticeps*)	81/92 (88%)	2e-41
GST	AJ629018	201	Glutathione S-transferase protein (*Nilaparvata lugens*)	77/77 (100%)	0

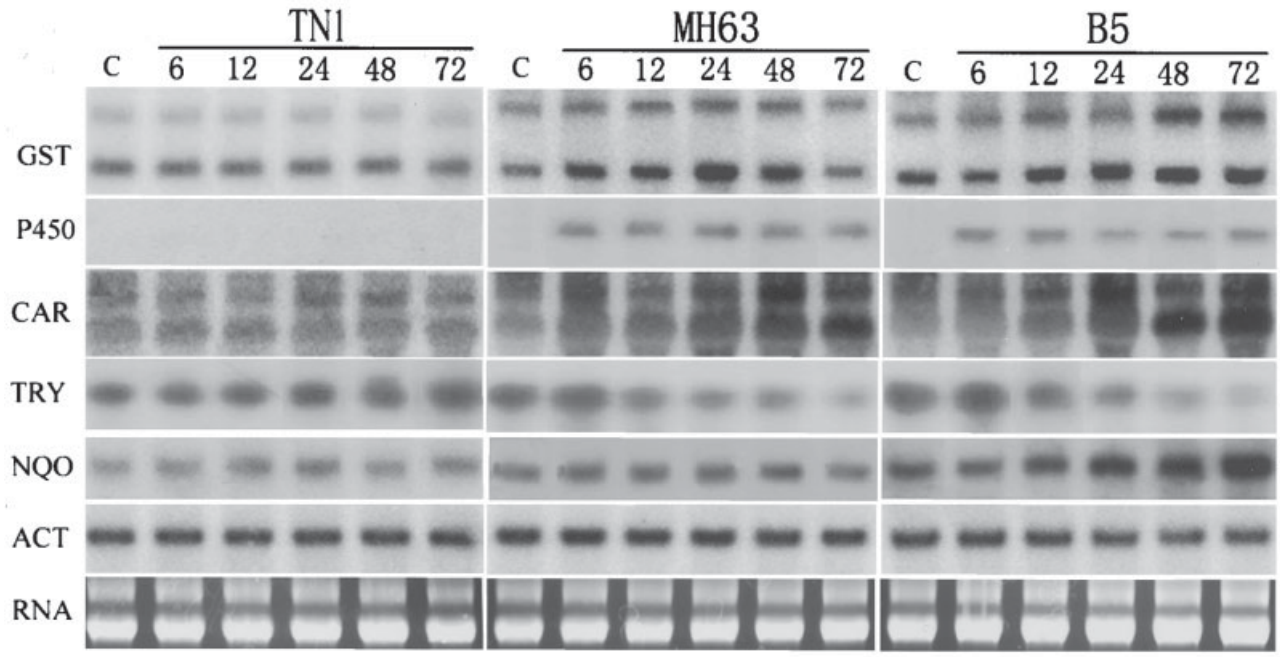

Fig. 2. Northern analysis of 4th instar nymphs of brown planthopper probing for P450, GST, CAR, TRY, and NQO mRNAs. Equivalent loading of total RNA in each lane was verified by rRNAs stained with ethidium bromide and the bands showing actin (ACT) transcripts hybridized with a 200-bp cDNA probe. TN1: Nymphs feeding on TN1 plants; B5: nymphs feeding on B5 plants; and MH63: nymphs feeding on MH63 plants. Nymphs were collected at the indicated time points (6, 12, 24, 48, and 72 h) for RNA isolation. The fourth instar nymphs at the time of starting the feeding experiment (0 h) were used as control (C).

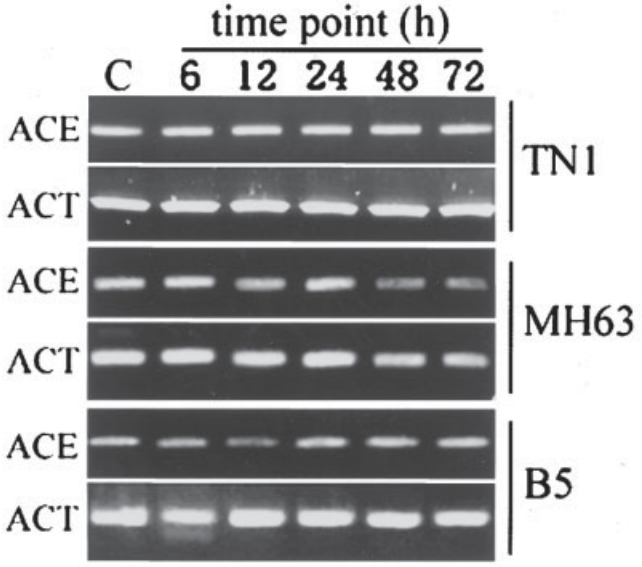

Fig. 3. RT-PCR confirmation of ACE gene expression in brown planthopper feeding on TN1, B5, and MH63 plants. Equivalent input of total RNA in each reaction was quantified by amplified β-actin (ACT) cDNA. For details, see Figure 2.

gene, NQO gene, and ACE gene are new ones cloned from BPH (Table 2).

Insect P450s metabolize hormones and pheromones but are best known for their roles in the metabolism of insecticides and host plant chemicals (Hung et al., 1997; Feyereisen, 1999). Most phytophagous insects encounter large amounts of predictable allelochemicals, and have relatively high P450-based metabolism towards such compounds. In this study, the expression of the P450 gene remained at a very low level in control insects feeding on susceptible rice TN1, but was elevated in BPH feeding on resistant rice B5 and MH63, suggesting there might be toxic allelochemicals in both rice varieties, which induced the P450 gene in a signaling cascade. This P450 gene is most likely involved in detoxifying allelochemicals from rice, although we cannot exclude that P450 may take part in other metabolic pathways involved in BPH on ingestion of resistant rice plants (Fig. 2).

CARs are enzymes hydrolyzing ester bonds in the presence of water. Since many insecticides and plant allelochemicals contain ester bonds, it is not surprising that the mechanism of insect resistance to insecticides and allelochemicals in many cases is caused by enhanced level of CARs. Hemming and Lindroth (2000) found that gypsy moth CAR activities were induced by phenolic glycoside, an allelochemical in aspen leaves. The expression pattern of the CAR gene in this work suggests certain allelochemicals in the sap of B5 and MH63 induced this gene in BPH (Fig. 2), and that there are more CAR molecules generated in BPH on B5 and MH63 plants than on TN1 plants. This result also implies that CAR is one of the early enzymes that appeared in BPH in response to ingesting the resistant rice plants. Esterases also are known for sequestering insecticides even if the hydrolysis is very low. Sequesterization also can be a survival mechanism in the presence of allelochemicals.

The final groups of enzymes that provide metabolic resistance are GSTs. They possess a wide range of substrate specificities, including endogenous substrates, such as reactive unsaturated carbonyls, reactive DNA bases, epoxides, and organic hydroperoxides produced in vivo as the breakdown products of macromolecules during periods of oxidative stress (Hayes and Pulford,, 1995). Thus, GSTs play a vital role in protecting tissues against oxidative damage and oxidative stress. Plant allelochemicals can induce increased GST production in insects (Leszczynski et al., 1994). In the present study, Northern analysis revealed that GST gene expression was enhanced in BPH feeding on B5 plants, suggesting that more oxidative stress and damage were imposed on BPH. Fluctuation of this gene expression in BPH feeding on MH63 suggests less oxidative stress affected BPH, and the gene expression needs to change relatively little to overcome the stress in such a case (Fig. 2).

Protease inhibitors (PIs) are undoubtedly among the most studied anti-herbivore proteins of plants, which tightly bind to proteases and thereby inhibit their activity (Richardson, 1991). The metabolic consequences for insect herbivores ingesting a diet with high concentrations of PI are thought to include a lack of available amino acids, which may lead to oversecretion of trypsin, further loss of sulfur amino acids (Ryan, 1990), and a decrease in trypsin mRNA levels (Gatehouse et al., 1997). TRY was apparently down-regulated in this study (Fig. 2), indicating that this gene was probably suppressed by PIs in the sap of B5 and MH63 plants to some extent, which coincided with BPH infestation that could induce protease inhibitor gene in rice plants (Zhang et al., 2004).

NQO is the first electron transport enzyme of the respiratory chain. It contributes to ATP synthesis. Studies revealed that some secondary metabolites from microbial and plant sources, such as rotenone and piericidin A, are important components that act on the respiratory chain by inhibiting NQO (Lümmen, 1998). NQO gene expression was enhanced in BPH feeding on B5, indicating there might be some compounds having a similar structure and function as rotenone or piericidin A generated in B5 plants, which might inhibit the respiratory chain enzymes in BPH to a certain degree. Alternatively, some stimuli from B5 plants might induce this gene by a series of signaling transductions. Contrary to this, there seemed no such components in TN1 and MH63 plants (Fig. 2).

ACEs are the well-known target enzymes of organophosphates, carbamates, and some plant chemicals such as monoterpenes. These toxins can bind to ACEs and prevent the enzymes from stopping the action of the neurotransmitter acetylcholine (Keane and Ryan, 1999). In this study, RT-PCR showed there were no changes in the expression pattern of ACE gene in BPH feeding on both resistant rice varieties, suggesting this gene in BPH was not influenced by resistant rice (Fig. 3). We are also interested in the sodium channel gene in BPH, but failed to clone the gene by using the RT-PCR method.

In summary, the expression profile of five genes was altered in BPH after ingesting two rice varieties with different resistance levels. Furthermore, as the expression of the GST and NQO genes was increased persistently in BPH feeding on B5, it appears that BPH was under more severe stress from B5 than that from MH63. These results suggest that

the five genes may play important roles in the defense response of BPH to resistant rice. The elucidation of the molecular dynamics and regulation of the genes will help in the design of effective control programs.

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